The current success in treating human immunodeficiency virus (HIV) infection and the global AIDS pandemic can be attributed, for the most part, to effective combination antiretroviral therapy, involving a cocktail of inhibitors directed against the retroviral protease and reverse transcriptase (RT). Inhibition of DNA polymerase function is achieved either directly through the incorporation of nucleoside analogues (NRTIs, for example azidothymidine) to prevent extension of the growing DNA chain, or allosterically by non-nucleosides (NNRTIs, for example nevirapine or efavirenz) that occupy a hydrophobic pocket in the vicinity of the active site and inhibit DNA synthesis without themselves being incorporated. Although not currently in clinical use, TSAO-T derivatives – which are highly-functionalised thymine analogues – target the interface between the p66 and p51 subunits, leading to dissociation of the p66/p51 RT heterodimer, while the indolopyridone inhibitor INDOPY-1 has been proposed to compete with the natural deoxynucleoside triphosphate substrates (2). As this armament of antiviral agents expands (see Figure 1), the absolute dependence on the ribonuclease H (RNase H) activity of HIV-1 RT for virus replication suggests that small molecules targeting this key function should be given consideration, especially if they can be demonstrated to potentiate the activity of those drug combinations currently in use.

The absolute dependence of HIV-1 Reverse Transcriptase – and thus HIV replication – on ribonuclease H activity suggests that small molecules targeting this function offer potential as anti-AIDS therapies, especially if they can be shown to potentiate the activity of those drug combinations currently in use.

FUNCTIONS OF RETROVIRAL RIBONUCLEASE H

What are the contributions of RT-associated RNase H during HIV replication and are they critical for virus propagation? Following infection, the single-stranded (+) RNA genome of the invading virus must be converted into a double-stranded DNA provirus suitable for insertion into the host chromosome by the HIV integrase protein (see Figure 2). Reverse transcription of the RNA template creates an RNA/DNA hybrid whose RNA strand must be removed prior to synthesis of the second (or (+)) DNA strand. RNase H activity of HIV RT can therefore be primarily seen as a mechanism whereby RNA of the RNA/DNA replication intermediate is removed by nonspecific hydrolysis. However, certain steps in the reverse transcription process require a greater degree of RNase H cleavage specificity and – as such – may be more sensitive to inhibition. One example is generating the 3' terminus of the polypurine tract (PPT) RNA primer from which (+) strand DNA synthesis is initiated. In contrast to the RNA/DNA replication intermediate, which is non-specifically hybridised, the all-purine nature of the PPT (and most likely additional structural features) renders it RNase H-resistant, thereby providing a 3' OH group for (+) strand, DNA-dependent DNA synthesis. As DNA synthesis proceeds over the recently created (+) strand
DNA template, the PPT must next be precisely removed in order to support the later step of proviral DNA integration. This is accomplished by RNase H-mediated cleavage between the PPT RNA and (+) strand DNA (defined as the PPT<>U3 junction).

Equally critical to successful HIV replication is the removal of the host-coded tRNA primer that is exploited by HIV to initiate (-) strand DNA synthesis – tRNA\textsubscript{Lys,3}. An early step of (+) strand DNA synthesis involves polymerisation over a small segment of the tRNA\textsubscript{Lys,3} primer, a consequence of which is that nascent (+) strand DNA is hybridised to a (-) DNA-tRNA chimera. In a manner analogous to PPT-primed (+) strand DNA synthesis, precise RNase H cleavage at the tRNA\textsubscript{Lys,3}-DNA junction liberates the primer, whose dissociation promotes a second strand transfer event and continued (+) strand DNA synthesis, eventually resulting in an RNA primer-free proviral DNA duplex. The notion that, within a series of non-specific hydrolytic events, two steps in the HIV reverse transcription process mandate exquisite selectivity of the RNase H active site suggests that small molecules that either globally affect enzyme activity, or target a specific stage in HIV replication by disrupting the relevant nucleoprotein complex, could be developed.

CURRENT RNase H INHIBITOR STATUS

Introducing a single Glu478 \(\rightarrow\) Gln478 mutation at the RNase H active site selectively destroys enzyme activity \textit{in vitro} and eliminates infectivity \textit{in vivo} when introduced into the context of infectious proviral DNA. While these combined observations illustrate the potential of RNase H as a therapeutic target, inhibitor development has been slow compared with the substantial array of protease and RT drugs. However, recent developments have shown promise with respect to identification of active site and 'off-site' RNase H inhibitors. While originally controversial, it is now accepted that RNase H-mediated hydrolysis of RNA/DNA hybrids is a two-metal-catalysed event, with a preference for Mg\textsuperscript{2+} as the divalent cation. Sequestering divalent metals in the active site would therefore seem an effective means of interrupting catalysis.

N-hydroxyimides developed at Hoffman-La Roche and the natural product β-thujaplicinol identified at the US National Cancer Institute (NCI) (see Figure 1) are two examples of such inhibitors that specifically target the RNase H active site, most likely binding metal via their three oxygen ligands. Although not shown in Figure 1, the diketo acid-based inhibitor 4-[5-(benzoylamino)thien-2-yl]-2,4-dioxobutanoic acid reported by Merck most likely functions by a similar mechanism. For these three classes of antagonist, inhibition of RNase H activity could be achieved \textit{in vitro} in the low to sub-micromolar range, while DNA polymerase activity was unaffected. More importantly, β-thujaplicinol was demonstrated to be considerably less active on human RNase H – indicating good selectivity. Although a low-resolution model for an N-hydroxyimide bound to the isolated RNase H domain has been obtained, there are currently no reports of a high resolution co-crystal structure of the RT/RNase H inhibitor complex, which would facilitate development of more potent and selective analogues. Our research on natural product RNase H inhibitors has indeed demonstrated that modification of the dihydroxypolypeptide scaffold increases potency.

Figure 1 is intended to illustrate the notion that several 'pockets' outside the DNA polymerase active site of HIV-1 RT can be targeted by small molecule antagonists, the most notable being the NNRTI binding site at the base of the p66 thumb subdomain. The crystal structure of HIV-1 RT and co-crystals containing DNA polymerase inhibitors or nucleic acid indicate that the thumb of the smaller p51 subunit provides support for the C-terminal RNase H domain of p66. It would therefore not be an unreasonable assumption that small molecules interacting with the p51 thumb might induce a conformational

Figure 2: RNase-H mediated events during RT-catalysed conversion of single-stranded RNA of the invading virus into integration-competent, double-stranded DNA

Individual steps in the reverse transcription process are indicated in blue, and a requirement for RNase H activity at that particular step in red. A more complete description of events can be found in (1).
change at the RNase H active site that alters the geometry of the catalytically critical divalent metals. While the exact mechanism remains to be established, we recently reported a novel class of RNase H inhibitors that possibly function via such a mechanism. The vinylogous ureas 2-amino-5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophene-3-carboxamide (NSC727447) and N-[3-(aminocarbonyl)-4,5-dimethyl-2-thienyl]-2-furancarboxamide (NSC727448) inhibit HIV-1 RNase H activity in vitro with IC₅₀s in the 2-5µM range. Protein footprinting by mass spectrometry suggested an interaction of NSC727447 with the p51 thumb as the likely mechanism of RNase H inhibition, a notion supported by molecular modelling studies (4). Thus, in a manner analogous to the NRTI/NNRTI combination of DNA polymerase inhibitors, a combination of ‘active site/off-site’ RNase H antagonists might be considered.

**NATURAL PRODUCT INHIBITORS**

Natural products continue to represent a unique and diverse source of biological material for molecular target elucidation, validation and drug discovery research. Since 1986, the Developmental Therapeutics Program of the NCI has acquired plants and marine organisms in over 25 tropical and subtropical countries worldwide. As of 2009, over 80,000 plant samples have been collected in Africa and Madagascar, Central and South America and Southeast Asia, while during the same time over 13,000 marine invertebrates and marine algae were collected, primarily from the Indo-Pacific and Australasia regions. Collectively, over 150,000 extract samples are available in 96-well plates in the NCI Natural Products Repository for screening and lead compound isolation. In addition to β-thujaplicinol and manicol – derived from the bark of the western cedar *Thuja plicata* and Guyanan tree *Dulacia guianensis*, respectively – our screening of NCI natural product extracts has identified dimeric lactones from *Ardisia japonica*, 1,3,4,5-tetragalloylapiitol from the African plant *Hylocladon gabunensis* and phenolic glycodides from *Eugenia hyemalis* as HIV-1 RNase H inhibitors (see Figure 3).

**DUAL RNase H/INTEGRASE INHIBITORS**

The RNase H domain of RT and the HIV-1 integrase protein are members of the superfamily of polynucleotidyl phosphotransferases, sharing an -Asp-Asp-35aa-Glu- active site motif. It is therefore conceivable that small molecule inhibitors directed against one HIV enzyme might retain activity against its mechanistically equivalent counterpart. As proof of this concept, the specificity of several derivatives of madurahydroxylactone, a secondary metabolite from the soil bacterium *Nonomuraea rubra*, for HIV-1 RNase and integrase was evaluated. In addition to identifying compounds with equal potency against both enzymes, selective inhibition of RNase H or integrase was also observed. Although the notion that two HIV enzymes can be targeted with a single compound remains to be validated in vivo, data with madurahydroxylactone derivatives at the very least suggests that systematic screening against both enzymes would be a fruitful strategy.

**PHARMACOLOGICAL INTERACTIONS BETWEEN RNase H AND DNA POLYMERASE INHIBITORS**

When targeting multiple inhibitors to different sites of a single, multi-functional enzyme such as HIV-1 RT, an important consideration is whether their effects are additive – that is, whether they function independently of each other, or whether they are synergistic, inducing a larger effect than each of the individual components. Less desirable is the possibility that targeting different sites might be antagonistic – that is, the inhibitor combination results in an effect that is smaller than each inhibitor alone. Early work from researchers at Merck indicated that activity of the diketo acid inhibitor 4-[5-(benzoylamino)thien-2-yl]-2,4-dioxobutanoic acid was enhanced in the presence of elevated concentrations of both NRTIs and NNRTIs (5). In support of this notion, we have also demonstrated synergy between the natural product RNase H inhibitor β-thujaplicinol and the natural product NNRTI calanolide A (6). While these preliminary observations are encouraging, the following section indicates the need to exercise caution when simultaneously targeting the DNA polymerase and RNase H functions of HIV-1 RT.

**WILL RNase H INHIBITORS INDUCE ENHANCED NRTI RESISTANCE?**

Recent reports have raised an important issue that inhibition of RNase H function might in fact be deleterious with respect to other RT inhibitors in clinical
use. In one study, inactivating mutations introduced into the RNase H domain were shown to correlate with enhanced AZT resistance; the underlying mechanism was that the increased 'residency time' of mutant RT at the primer terminus allowed more time for pyrophosphorylytic removal of the chain terminator and resumption of DNA synthesis (7). Subsequent to this, examination of the RNase H coding region of RT from drug-resistant isolates indicated a correlation between increased NRTI resistance and mutations in the connection subdomain or RNase H domain (8). While this issue should not be overlooked, it is important to bear in mind that as inhibitors targeting the activities of HIV proteins and enzymes emerge, this will most likely require altering the components of combination antiretroviral therapy. In support of further development of RNase H inhibitors, our recent studies have demonstrated that mutations in the connection subdomain close to the RNase H active site result in sensitisation to vinylogous ureas (4). Stated differently, should an RNase H inhibitor with good potency, selectivity and pharmacokinetics emerge, eliminating AZT from combination therapy could be considered.

CONCLUSION

The absolute requirement of RNase H activity for HIV replication provides yet another target for therapeutic intervention. Crystallographic and mechanistic studies promise to reveal additional platforms for the development of novel inhibitors. While identification of potent RNase H inhibitors is in its infancy, selectivity is of paramount importance, since inhibition of human RNase H was shown to be associated with impaired mitochondrial DNA synthesis and a lethal embryonic defect in mice. Initial studies with hydroxylated tropolones provide encouraging evidence that this selectivity can indeed be achieved. Secondly, inhibiting RNase H function may also affect susceptibility to other classes of drugs; in other words, RNase H inhibitors should not compromise NRTIs, NNRTIs and integrase drugs currently in clinical use in order to provide maximal benefits from future combination therapy. In the short term, biochemical studies addressing their mechanism of action are warranted. Finally, demonstrating antiviral activity and localising resistance-conferring mutations to the RNase H domain will be required to validate RNase H as a therapeutic target.

References


Suhman Chung is a Visiting Fellow in the RT Biochemistry Section of the HIV Drug Resistance Program. His current research focuses on lead identification and optimisation of small molecule inhibitors that specifically target RNase H activity of HIV reverse transcriptase with emphasis on identifying active site and allosteric inhibitors. Email: chungs2@mail.nih.gov

Jason Rausch is a Staff Scientist in the HIV Drug Resistance Program, National Cancer Institute. Prior to this, Jason was Research Associate in the Department of Biochemistry, Case Western Reserve University, Cleveland, OH. His principle research interests are in characterising HIV-related protein-nucleic acid interactions. Email: rauschj@mail.nih.gov

Stuart Le Grice is Chief of the RT Biochemistry Section, HIV Drug Resistance Program, National Cancer Institute, Frederick, MD, and Head of the Center of Excellence in HIV/AIDS and Cancer Virology. His research interests include understanding how long range RNA interactions control virus replication, and identifying novel antiviral agents. Email: legrices@mail.nih.gov